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Effect of Extraction Method on Antioxidant Determination in Produce by Differential Pulse Voltammetry

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Due primarily to the many health benefits associated with a diet high in antioxidants, numerous assays have been developed to determine antioxidant content of foods. However, most are time-consuming and costly. This paper presents an electrochemical technique as a simple, sensitive and cost-effective alternative to traditional assays, and uses the presented technique to determine the optimal method for the extraction of antioxidants from produce samples. In this study, fresh carrot, tomato, rhubarb, raspberry, strawberry, red bell pepper, green bell pepper and yellow bell pepper samples were subjected to three different extraction methods: aqueous extraction, using phosphate buffer as the extraction solvent; organic extraction, using acidified methanol as the extraction solvent; and solventless extraction, using a Breville® home juicer. Extracts were then analyzed by differential pulse voltammetry (DPV). Differences in the potential and area of the principal peak, indicative of antioxidant activity and capacity respectively, were analyzed to determine the effect of extraction method on antioxidant measurement. Aqueous extraction was found to provide the most effective extraction relative to antioxidant activity, while organic extraction was most effective relative to antioxidant capacity. Limits of detection and quantitation were 1.9×10^{-4} mol/L and 6.5×10^{-4} mol/L, respectively. This study showed DPV to be a sensitive and effective technique for antioxidant determination.

Keywords: antioxidant activity; antioxidant capacity; ascorbic acid; differential pulse voltammetry; extraction

ABBREVIATIONS: ascorbic acid (AA); cyclic voltammetry (CV); differential pulse voltammetry (DPV); limit of detection (LOD); limit of quantitation (LOQ); microwatt (μW); nanowatt (nW); reactive oxygen species (ROS)

1. INTRODUCTION

Though vital to survival, oxygen is deleterious to the human body. During cellular respiration, reactive oxygen species (ROS), a type of free radical, are produced as by-products of ATP production [1, 2]. These species induce cellular damage through radical oxidation of biological components, including proteins, nucleic acids, and lipids. This oxidative stress can contribute to numerous diseases, including cancer, Parkinson's disease, Alzheimer's disease, multiple sclerosis, diabetes mellitus, rheumatoid arthritis and arteriosclerosis [3–7]. Additionally, oxidative stress has been linked to the degenerative processes associated with aging [3–6]. Antioxidants, compounds which prevent or mitigate oxidative stress, have thus been the focus of significant nutritional and medical study within recent years.

An antioxidant is a species capable of donating an electron to an oxidant, such as a ROS, thereby neutralizing its capacity to induce oxidative stress. Antioxidants can be endogenous or exogenous [5, 8]. Endogenous antioxidants exist naturally within the body while exogenous antioxidants are derived primarily from diet. Vitamin C, vitamin E, β-carotene, flavonoids, isoflavones, anthocyanins and polyphenols are all examples of exogenous antioxidants [2, 5, 9].

Due to the role antioxidants play in reducing oxidative stress, increased consumption of foods high in exogenous antioxidants has been associated with many potential health benefits [6]. Consequently, numerous qualitative and quantitative techniques have been developed to evaluate the antioxidant capacity (thermodynamic efficiency of an antioxidant) and activity (reaction kinetics of an antioxidant, unique to a specific species) of foods and supplements [10–12]. The majority of these techniques rely upon spectrophotometric chemical assays which indirectly measure antioxidant capacity based on an observed shift in sample absorbance. However, these assays are often time-consuming, non-specific and costly, and the results from different assays are usually not comparable [6, 11, 12].

Recently, several studies have established electrochemical techniques as simple, cost-effective alternatives to traditional antioxidant assays [13, 14]. Cyclic voltammetry (CV), for instance, has been successfully used to determine the antioxidant capacity of blood plasma [8], mushroom extracts [15], tea infusions [10], Algerian dates [16], fruit samples [4, 12] and wines [17]. However, the usefulness of CV for quantitative analysis of organic samples is limited by the adsorption phenomena which can occur at the electrode surface [15, 18].

Differential pulse voltammetry (DPV) has also been used for antioxidant characterization, and particularly for quantitative determinations. Unlike CV, the pulsed nature of DPV enables the discrimination of adsorption phenomena, thus allowing for more sensitive quantitative analysis [15, 18]. For this reason, DPV is becoming an increasingly common technique for organic analysis, and has been successfully used to determine ascorbic acid in tablets and fruit juices [19], the phenolic content of wines [20], and even the presence of the antioxidant 2,6-ditertbutyl-4-methylphenol in jet fuels [21]. However, no work in the literature reports the use of DPV to analyze the impact of extraction method on antioxidant determination. The principal aim of this study was thus to use DPV to determine the optimal extraction method for exogenous antioxidants present in produce.

2. EXPERIMENTAL

2.1. Reagents

Sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, *o*-phosphoric acid, hydrochloric acid, anhydrous ethanol and methanol were purchased from Fisher Scientific. *L*-ascorbic acid was obtained from Aldrich and potassium chloride from J. T. Baker. All reagents were reagent grade purity.

2.2. Fruit and vegetable selection and preparation

All fruit and vegetable samples were obtained and analyzed during June and July 2013. Fruits and vegetables were chosen to represent a wide range of botanical classifications, namely: vegetable root (carrot), vegetable fruit (tomato), vegetable petioles (rhubarb), aggregate fruit (raspberry), accessory fruit (strawberry), and berry fruit (red, green and yellow bell pepper).

To remove surface contaminants, all samples were rinsed well with tap water and dried prior to extraction. In all cases, only the traditionally consumed portion of each fruit/vegetable was used for analysis.

2.3. Extraction methods

Three different extraction methods were used during the course of study: organic extraction, aqueous extraction, and solventless extraction. In all cases, pH 2.0 phosphate buffer was used either as the extraction solvent or to dilute extracted antioxidants prior to electrochemical analysis. Phosphate buffer was prepared from 0.04 mol/L sodium phosphate dibasic heptahydrate, 0.06 mol/L sodium phosphate monobasic monohydrate and 0.1 mol/L KCl, and brought to the desired pH using 6 mol/L *o*-phosphoric acid.

2.3.1. Organic extraction

Finely chopped sample (25 g) was placed in a 250-mL Erlenmeyer flask and diluted with methanol (60 mL) and 0.3 mol/L HCl (10 mL). The flask was sparged with nitrogen gas, sealed, and placed in the dark. Samples were stirred vigorously for 24 h, after which the remaining pulp was removed by suction filtration through Whatman No. 4. filter paper. Methanol was removed from the filtrate by evaporation under reduced pressure, and the resultant residue diluted with 0.1 mol/L phosphate buffer at pH 2.0 (50 mL).

2.3.2. Aqueous extraction

Sample (25 g) was diluted with 0.1 mol/L phosphate buffer at pH 2.0 (25 mL) and blended using a Magic Bullet® blender until no visible solids remained (blending time ≈ 1 min). The resultant

mixture was suction filtered through Whatman No. 4. filter paper, using an additional 25 mL buffer for rinsing.

2.3.3. Solventless extraction

A whole segment of sample (25 g) was blended using the Breville® Juice Fountain Plus Two-Speed model JE98XL (a typical home juicer) on the low setting. The extracted juice was then diluted with 0.1 mol/L phosphate buffer at pH 2.0 (50 mL).

2.4. Instrumentation and electrode preparation

DPV analysis was performed using a Pine Instrument Company bipotentiostat model AFCBP1 and AfterMath™ software. All tests were carried out using a standard three-electrode cell, a Pt counter electrode, a Ag|AgCl reference electrode, and a glassy-carbon working electrode. Prior to each test, the working electrode was polished successively with aqueous slurries of 1.0, 0.3 and 0.05 micron Buehler Micropolish II® alumina powder on a Buehler Microcloth® pad. Following each polish, the electrode was rinsed well with distilled water and sonicated in distilled water for 5 minutes. After the final polish, the electrode was sonicated in distilled water for 5 min, followed by sonication in anhydrous ethanol for 5 min. The electrode was then rinsed well with distilled water and used immediately for testing. Prior to each test, the Pt counter electrode was similarly polished on a wetted Buehler Microcloth® pad and rinsed with distilled water.

2.5. Sampling plan

Three randomly selected samples of each fruit/vegetable type (e.g. three carrot stalks, three boxes of raspberries) were analyzed. To ensure consistency of matrix composition so as to allow for the comparative analysis of extraction method impact on antioxidant determination, each of the three samples of each produce type was then subjected to organic extraction, aqueous extraction and solventless extraction (Fig. 1). Immediately following their preparation, all extracts were sparged with nitrogen gas for 2 minutes, then analyzed by DPV. Each extract was analyzed twice to minimize random error; the average of the two values was used as the data point for that sample.

2.6. Differential pulse voltammetry

DPV characterization was carried out between 200 and 900 mV, using the following parameters: height = 50 mV; width = 100 ms; period = 200 ms; increment = 1 mV; pre and post-pulse width = 3 ms; electrode range = 10 µA for carrot, tomato, rhubarb, raspberry, strawberry; electrode range = 30 µA for red, green and yellow bell peppers. For each extract, the principal peak potential and the total peak area from 200 to 900 mV was determined; for area measurements, the baseline was set as the minimum current value within the 200 to 900 mV range.

2.7. Ascorbic acid calibration curve

To allow the expression of data in ascorbic acid (AA) equivalents, a calibration curve of peak area as a function of AA concentration was prepared using the same parameters as adopted for sample analysis ([AA] = 5.0×10^{-3} to 7.9×10^{-5} mol/L) (Fig. 2). Standards were prepared using 0.1 mol/L phosphate buffer at pH 2.0 as a solvent. The curve was also used for method validation (Table 1). The accuracy and precision of the calibration curve were assessed and the limit of detection (LOD) and limit of quantitation (LOQ) calculated:

LOD = 3 *s/m*

LOQ = 10 *s/m*

Where *s* is the standard deviation of the intercept (n = 3) and *m* is the slope [19].

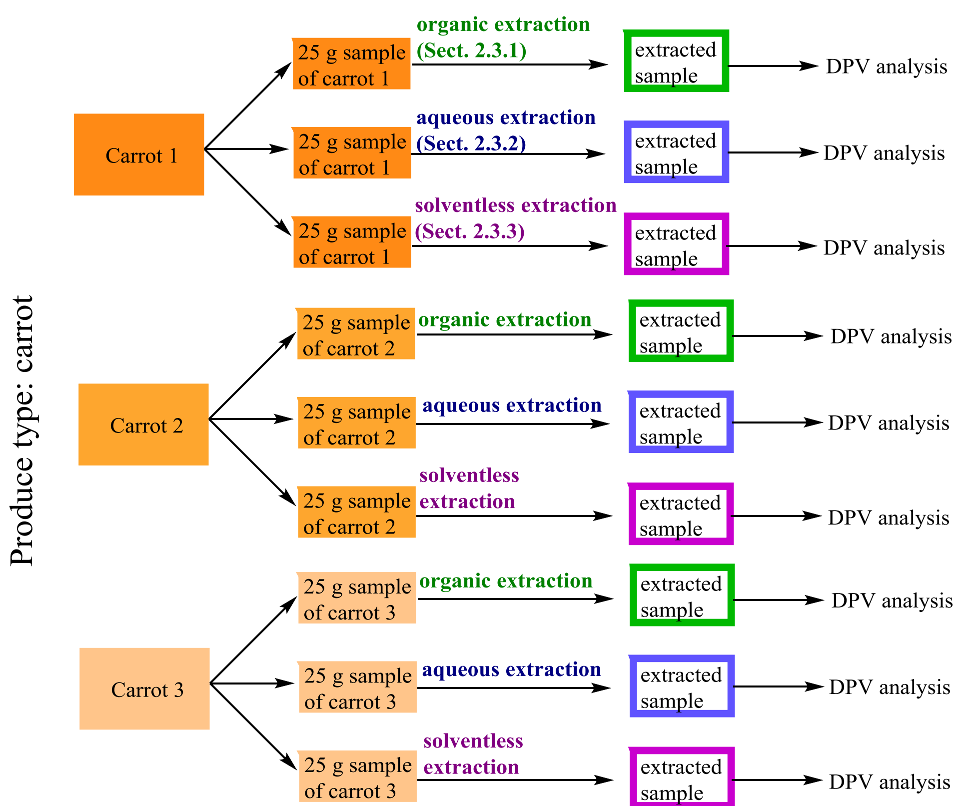

Figure 1. Schematic representation of sampling plan. For each produce type, three independent samples (carrot 1, 2 & 3) were each subjected to each of the three extraction methods (organic, aqueous, solventless) and the extracts analyzed by DPV.

2.8. Data analysis

All results are presented as the calculated mean ± standard deviation. Results were analyzed using Microsoft® Excel 2013 and AfterMath™ software. Comparative analysis of extraction methods (organic vs. aqueous, organic vs. solventless, and aqueous vs. solventless) for each produce sample

was performed using two-tailed paired-Student's t-tests. Results were deemed to be significant at $p < 0.05$.

To express antioxidant concentration in AA equivalents, the equation of the calibration curve (Fig. 2) was used:

$$y = 684.24x + 0.1479$$

Where y is the measured peak area in microwatts (μW) and x is the antioxidant concentration in AA equivalents (mol/L). The [AA] of the original fruit samples (in mg AA per 100 g sample) was then calculated using the extraction dilution factor (25 g sample in 50 mL buffer).

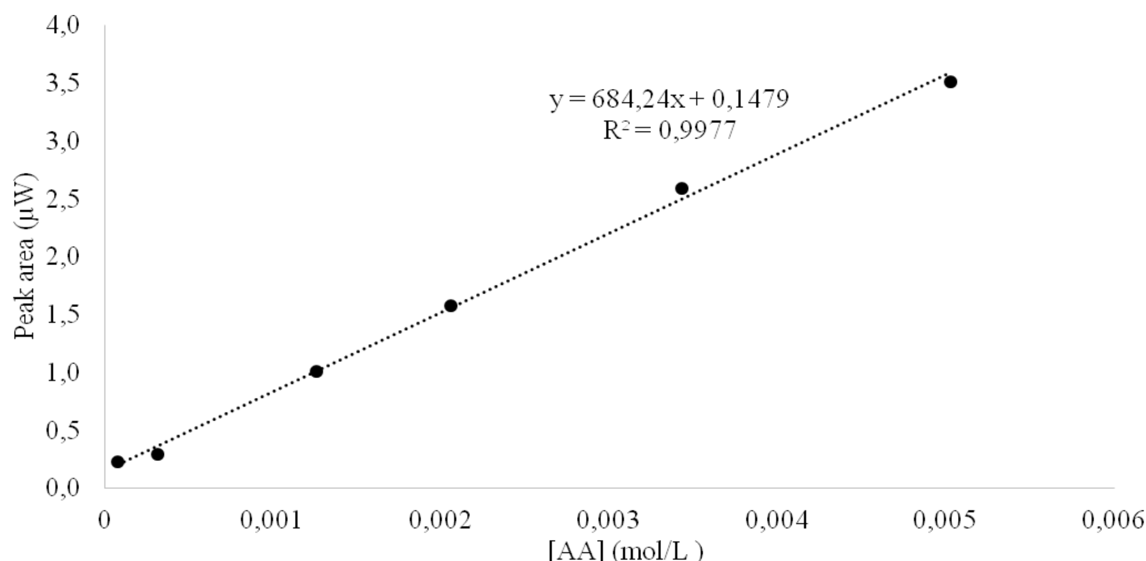

Figure 2. Calibration curve expressing voltammogram peak area (in microwatts, μW) as a function of ascorbic acid (AA) concentration; n = 6, [AA] = 5.0×10^{-3} to 7.9×10^{-5} mol/L.

3. RESULTS AND DISCUSSION

3.1. Differential pulse voltammetry overview

Representative voltammograms of the eight produce types analyzed are shown in Figure 3. All voltammograms exhibited an intense, well-defined principal peak in the 340 to 560 mV region (Table 1). This peak can likely be attributed to the irreversible oxidation of flavonoids, benzoic acid and cinnamic acid antioxidant derivatives [22]. At pH 2.0, many phenolic compounds exhibit an oxidation potential within the 300 to 600 mV region, including: anthocyanins (440-590 mV), protocatechuic acid (590 mV), quercetin (440 mV), rutin (510 mV) and (+)-catechin (530 mV), among many others [10, 22–25]. Other non-phenolic antioxidants, such as gallic acid (460 mV), also possess an oxidation potential in this region at pH 2.0, and thus may have also contributed to the principal peak [4, 10, 15]. The observed peak potential for each produce sample is ultimately determined by the sample's relative content of high and low oxidation potential antioxidants.

Table 1. Potential (E_p) and area (A, measured in nanowatts) of principal peak in differential pulse voltammograms of analyzed fruits and vegetables (n = 3).

	Aqueous extract		Solventless extract		Organic extract	
Sample	E_p (mV)	A (nW)	E_p (mV)	A (nW)	E_p (mV)	A (nW)
Carrot	476±1	688±202	484±1	345±28	491±4	614±80
Tomato	451±4	704±48	461±5	489±30	493±11	730±135
Yellow bell pepper	455±36	3284±91	458±42	2351±485	537±18	3215±456
Red bell pepper	445±40	3466±558	470±20	2801±658	558±15	3733±494
Green bell pepper	462±45	3264±510	489±22	2425±480	546±28	3442±828
Rhubarb	492±8	1567±379	479±10	1251±271	471±16	1329±99
Strawberry	374±1	1466±93	345±82	897±189	488±45	2478±86
Raspberry	415±3	1423±103	414±1	700±61	471±8	1305±68

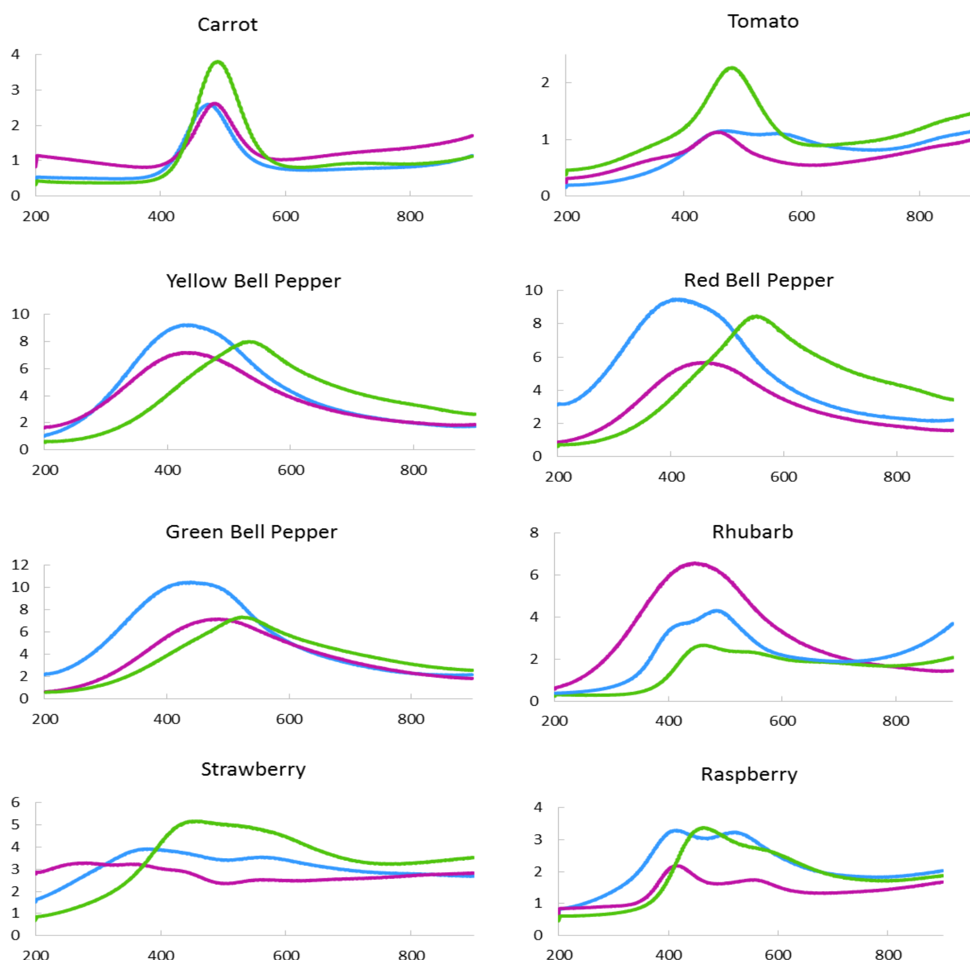

Figure 3. Representative differential pulse voltammograms for aqueous (blue), solventless (purple) and organic (green) extracts of produce samples, showing current (µA, y-axis) as a function of applied voltage (mV, x-axis).

When comparing the experimental data to that found in the literature, it is important to note that a one unit decrease in pH leads to a positive potential shift of approximately 35 mV, while a one unit

increase in pH leads to a negative shift of 35 mV [10]; the above listed potential values were adjusted to the relevant pH using this rule.

It should also be noted that additional peaks were observed above 560 mV for some samples. However, such secondary peaks were consistently much less intense than the principal peak. These peaks may indicate the presence of low concentrations of higher oxidation potential antioxidants, such as salicylic acid (1100 mV), vanillic acid (900 mV) and coumaric acids (850-950 mV) [22].

3.2. Determination of antioxidant activity

Antioxidant activity is a kinetic measurement of the reaction rate of a particular antioxidant with its target oxidant [11, 12, 14, 26]. In DPV, antioxidant activity is determined by peak potential, with a lower peak potential indicating a higher activity [3, 13]. Differences in the relative concentration of low oxidation potential (high activity) and high oxidation potential (low activity) antioxidants in the analyzed samples will have affected the location of the principal peak. For instance, a sample containing a large proportion of antioxidants possessing a high activity will have a relatively low principal peak potential. Analysis of peak potential thus allows for the meaningful comparison of the total antioxidant activity of samples across extraction method.

In all but one of the performed analyses, samples extracted under aqueous conditions demonstrated a significantly lower principal peak potential than samples subjected to organic extraction (carrot, $p = 0.011$; tomato, $p = 0.030$; yellow bell pepper, $p = 0.017$; red bell pepper, $p = 0.018$; green bell pepper, $p = 0.018$; strawberry, $p = 0.048$; raspberry, $p = 0.003$) (see Table 1 for peak potentials). The only exception to this trend was rhubarb, which demonstrated a significantly lower potential under organic extraction conditions ($p = 0.042$), possibly due to the fibrous nature of rhubarb which prevented effective blending during aqueous extraction. This trend indicates that aqueous conditions enable a more effective extraction of low oxidation potential antioxidants than does an organic environment. A possible explanation is that the low oxidation potential antioxidants most commonly found in fruits and vegetables (e.g. ascorbic acid, flavonoids, some polyphenols) are predominantly hydrophilic and so are better suited to extraction in an aqueous medium [2]. The use of aqueous extraction in high activity antioxidant determination of food samples could thus potentially increase detection sensitivity.

Similarly, in all but two cases, samples subjected to solventless extraction (using the Breville juicer) demonstrated significantly lower oxidation potentials than did organically extracted samples (carrot, $p = 0.042$; tomato, $p = 0.046$; yellow bell pepper, $p = 0.029$; red bell pepper, $p = 0.004$; green bell pepper, $p = 0.006$; raspberry, $p = 0.008$). This further confirms the ineffectiveness of organic conditions in the extraction of low oxidation potential antioxidants.

Regarding the comparison of the aqueous and solventless extraction methods, significant differences were observed for only two samples (carrot, $p = 0.0005$; tomato, $p = 0.012$); in both cases, aqueous extraction proved the most efficient. However, there is insufficient evidence to suggest that aqueous extraction is more effective than solventless extraction.

3.3. Determination of antioxidant capacity

Antioxidant capacity is a measurement of the thermodynamic efficiency of an antioxidant and is directly related to the concentration of antioxidants present within a sample [11, 12, 26]. Antioxidant capacity can be electrochemically determined from either the peak current or peak area of a voltammogram, with a greater current/area indicating a greater antioxidant capacity [8, 10].

In contrast to the determination of antioxidant activity, in which organic extraction proved the least effective (section 3.2.), organic extracts repeatedly demonstrated significantly higher antioxidant capacities than either solventless or aqueous extracts (solventless vs. organic: carrot, $p = 0.050$; strawberry, $p = 0.003$; raspberry, $p = 0.004$; aqueous vs. organic: strawberry, $p = 0.009$). In no case did aqueous extraction or solventless extraction demonstrate a significantly higher antioxidant capacity than organic extraction. This result, combined with the noted ineffectiveness of organic extraction relative to antioxidant activity, may indicate that organic solvents lead to ineffective extraction of low oxidation potential antioxidants (hence the relatively low activities observed in organic extracts) but effective extraction of higher oxidation potential antioxidants. However, a more detailed characterization of the composition of the extracts would need to be performed before such a relationship could be conclusively determined.

Regarding the comparison of aqueous and solventless extracts, no consistent trend was observed, though aqueous extracts did demonstrate significantly higher capacities for both tomato ($p = 0.040$) and raspberry ($p = 0.013$) samples.

3.4. Quantitative determination of antioxidant content

Table 2. Total antioxidant content of samples. All values are presented as ascorbic acid equivalents ($n = 3$).

	[AA] (mg AA/100 g sample)		
Sample	Aqueous extract	Solventless extract	Organic extract
Carrot	27.8±10.4	10.2±1.5	24.0±4.1
Tomato	28.6±2.5	17.6±1.5	30.0±7.0
Yellow bell pepper	161.4±4.7	113.4±25.0	157.9±23.5
Red bell pepper	170.8±28.7	136.6±33.9	184.5±25.4
Green bell pepper	160.4±26.3	117.2±24.7	169.5±42.6
Rhubarb	73.0±19.5	56.8±13.9	60.8±5.1
Strawberry	67.9±4.8	38.6±9.7	119.9±4.4
Raspberry	65.6±5.3	28.4±3.1	59.5±3.5

Using the prepared AA calibration curve (Fig. 2), measured peak areas were used to express the total concentration of antioxidants present in the analyzed samples as mg AA per 100 g of sample. Results are shown in Table 2.

Overall, bell pepper samples showed the highest antioxidant content (113.4-184.5 mg/100 g), and carrots and tomatoes the lowest (10.2-30.0 mg/100 g), with raspberries and rhubarb being

intermediary (28.4-73.0 mg/100 g). The highest antioxidant content was observed in red bell peppers subjected to organic extraction (184.5 mg/100 g), while the lowest was seen in carrots subjected to solventless extraction (10.2 mg/100 g). For all produce types analyzed, samples subjected to solventless extraction demonstrated the lowest antioxidant content of the three extraction methods evaluated. No consistent trend was observed between aqueous and organic extraction in terms of quantifying antioxidant content.

3.5. Method validation

The ascorbic acid calibration curve was also used for method validation, adapted from the procedure used by Yilmaz et al. [19]. Validation parameters are shown in Table 1. A linear correlation between peak area and concentration was observed over the entire concentration range analyzed (5.0 x 10^{-3} mol/L to 7.9 x 10^{-5} mol/L). The method demonstrated adequate sensitivity, as indicated by the low relative standard deviation (% R.S.D.) of the slope (2.42 %). The method also possessed adequate repeatability, as indicated by the low % R.S.D. of the peak potential (2.54 %), peak height (4.62 %) and peak area (8.58 %), as determined from three independent analyses of a 7.85 x 10^{-5} mol/L ascorbic acid standard. Quantitative validation was determined via the limit of detection (LOD) and limit of quantitation (LOQ), found to be 1.9 x 10^{-4} mol/L and 6.5 x10^{-4} mol/L respectively.

Table 3. Validation parameters of DPV method. Parameters derived from calibration curve of ascorbic acid in 0.1 mol/L phosphate buffer at pH 2.0 at a glassy carbon electrode.

Validation Parameter	Measured Value
Peak potential (mV)	191.5±4.9
Linear concentration range (mol/L)	5.0 x 10^{-3} - 7.9 x 10^{-5}
Correlation coefficient, *r*	0.999
Slope (µW L/mol)	684±17
% R.S.D. of slope	2.42
Intercept (µW)	0.15±0.04
Number of concentrations analyzed (n)	6
LOD (mol/L)	1.9 x 10^{-4}
LOQ (mol/L)	6.5 x 10^{-4}
Repeatability of peak potential, height and area (% R.S.D.) based on three independent runs	2.54 % (potential), 4.62 % (height) and 8.58 % (area)

4. CONCLUSIONS

Due to the health benefits associated with the consumption of antioxidants, the determination of antioxidants in food samples is one of the fastest growing fields of electroanalytical chemistry and food science. This study demonstrates the successful use of differential pulse voltammetry (DPV) to determine the optimal extraction conditions for antioxidants in produce relative to antioxidant activity and capacity. Aqueous extraction conditions led to the most effective extraction relative to antioxidant

activity, while organic extraction was most effective relative to antioxidant capacity. The results showed that DPV is a highly sensitive and precise technique for antioxidant determination in produce samples.

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